IMMUNOASSAY OF UNCONJUGATED ESTRIOL IN SERUM OF PREGNANT WOMEN MONITORED BY CHEMILUMINESCENCE

W.Klingler, O.Haupt, G.v.Postel and R.Knuppen

Institut für Biochemische Endokrinologie der Medizinischen Hochschule Lübeck, Ratzeburger Allee 160, D-2400 Lübeck

Received: 7-6-83

ABSTRACT

6-Oxoestriol-6-(O-carboxymethyl)oxime-aminobutylethyl-isoluminol conjugate was synthesized. This luminogenic estriol derivative enabled us to develop a solid phase immunoassay method for the determination of unconjugated estriol in serum of pregnant women by the measurement of the bound estriol-isoluminol conjugate upon oxidation with a hydrogen peroxide/microperoxidase system. The sensitivity of the assay was 700 pmol/l. Results obtained by radioimmunoassay and the described method showed good agreement ($r = 0.95$). The chemiluminescent method is applicable in the routine measurement of unconjugated estriol.

INTRODUCTION

The determination of estriol concentrations in maternal blood is an important parameter for monitoring the well-being of the fetus and the function of the placenta. Estriol in serum is commonly determined by radioimmunoassay; either total estriol is measured after enzymatic hydrolysis or the unconjugated steroid is analyzed after ether extraction or directly without any extraction procedure. Although the radioimmunoassay procedures have many advantages, they pose some problems especially with regard to the disposal of radioactive waste. Alternative methods like enzyme immuno-

assays (1,2), fluorescence immunoassays (3,4) or electron-spin immunoassays (5) have been developed to avoid the disadvantages of the radioactive compounds. Experiments in different laboratories including ours have shown that chemiluminescence could be an alternative to radioactive labelling, too (6-11).

In this paper we describe a chemiluminescence immunoassay for quantitation of conjugated estriol in serum of pregnant women without an extraction procedure.

EXPERIMENTAL

Materials, solutions and miscellaneous. Estriol, estrone, estradiol-17β, cortisol and progesterone were purchased from Merck, Darmstadt, FRG; estriol-3-sulphate, estriol-3-glucuronide and estriol-16α-glucuronide from Sigma, München, FRG; 16-epi-estriol and 17-epi-estriol from Paesel, Frankfurt, FRG; 17-hydroxyprogesterone from Fluka, Ulm, FRG; microperoxidase (MP11) from Sigma, München, FRG; carboxy methoxylamine hemihydrochloride from EGA-Chemie, Steinheim, FRG; precoated silica gel thin layer chromatography plates (Polygram Sil G/UV 254) from Machery and Nagel, Düren, FRG; precoated silica gel thin layer chromatography plates (silica gel 60 F254) and all other chemicals from Merck, Darmstadt, FRG.

Anti-estriol antibodies raised in rabbits against 6-oxoestriol-6-(O-carboxymethyl)oxime-bovine serum albumin and fixed to solid particles ("Amerlex") were kindly provided by Amersham International, Amersham, UK. The concentration of this particle suspension was identical to that of the Amerlex Radioimmunoassay kit but the suspension was not colour-coded.

Stock solutions of steroids were prepared in ethanol, and just prior to use, diluted to the desired concentrations in assay buffer. Microperoxidase was dissolved at 1 mg/ml in Tris-HCl buffer (0.01 M, pH 7.4); this stock solution could be used within 6 months. Just prior to use, 0.04 ml of this solution was diluted with 8 ml of borate buffer (0.075 M, pH 8.6) yielding a 2.5 µM enzyme solution. The oxidant solution was prepared by adding 1 ml of 35 % hydrogen peroxide solution to 114 ml Tris-HCl buffer (0.01 M, pH 7.4).

Melting points were determined with a microscope hot-stage and are uncorrected. Infrared spectra using pressed KBr

discs were recorded with a Beckman Acculab 4 spectrometer. Light emissions were measured with a Biolumat LB 9500 (Laboratorium Prof. Berthold, Wildbad, FRG). The pump system of the automatic injection of the luminometer was equipped with teflon tubes to avoid contact between oxidant solution and metal.

Synthesis of 6-oxoestriol-6-(O-carboxymethyl)oxime. 6-Oxoestriol (0.725 g) (15) was dissolved in 50 ml of ethanol. Fifty-five ml of 1 M sodium acetate solution and 1 g of carboxymethoxylamine hemihydrochloride were added and refluxed for 4 h. Ethanol was evaporated *in vacuo* and the remaining aqueous phase was adjusted to pH 2 with 1 M HCl. This acidified phase was extracted three times with ethyl acetate. The combined organic layers were washed with water until neutral and evaporated *in vacuo*. The residue was redissolved in saturated sodium bicarbonate solution and extracted three times with ether to remove residual 6-oxoestriol. The remaining aqueous phase was adjusted to pH 2 and extracted three times with ethyl acetate. The ethyl acetate was washed with water and evaporated *in vacuo*. The semicrystalline residue was crystallized from methanol/water yielding white needles (0.9 g, mp. 169-170°C). Analytical data were identical to those published by Kuss *et al.* (14).

Synthesis of 6-oxoestriol-6-(O-carboxymethyl)oxime-aminobutylethyl-isoluminol (E3-6-CMO-ABEI). 6[N-(6-aminobutyl)-N-ethyl]amino-2,3-dihydrophthalazine-1,4-dione ("ABEI") was synthesized according to the method of Schroeder *et al.* (12). 6-Oxoestriol-6-(O-carboxymethyl)oxime (250 mg) was suspended in 7 ml of dry dioxane and 0.5 ml of tri-*n*-butylamine was added. The suspension was cooled to 11° and 0.22 ml of isobutyl chloroformate was added. The solution turned clear and it was stirred for 60 min at 11°. Aminobutylethyl-isoluminol (280 mg) was dissolved in a mixture of 31 ml of dioxane and 31 ml of water and the pH was adjusted to 8.5 at 0°C. The mixed anhydride was added dropwise to the stirred ABEI solution. During this procedure the pH was kept between 8.3 and 8.5 with sodium hydroxide solution (0.3 M). The solution was stirred for 3.5 h at 0°C. After adjusting the pH to 7.0 the solution was diluted with 200 ml of water and the solid material was filtered off and was dried *in vacuo*. Yield: 420 mg of solid material melting at 145-149°C. The product was identified by IR (3360 cm^{-1}, νO-H; 1765, νC=O) and elemental analysis. The proposed structure of the synthesized molecule is shown in Fig. 1.

Before use, 3 µg of this tracer substance was purified by thin-layer chromatography on silica gel using toluene/chloroform/methanol (40:40:20) as solvent system. E_3-6-CMO-ABEI (Rf = 0.15) was extracted with methanol from the plate and diluted with phosphate buffer (0.01 M, pH 6.2) to the final dilution of 40 pg/0.1 ml.

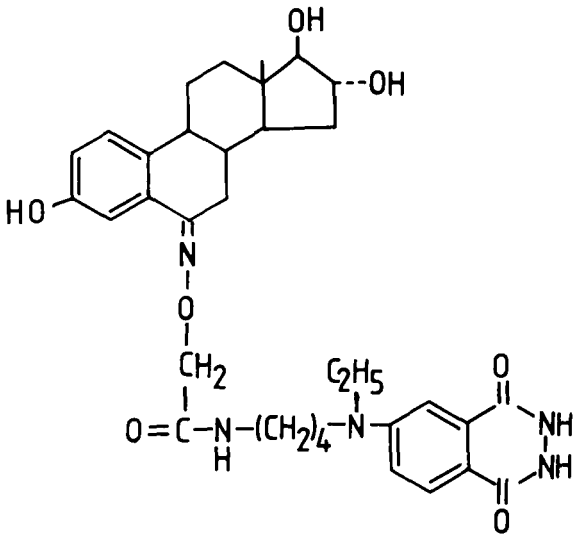

Fig. 1: Proposed structure for estriol-6-carboxymethyloxime-aminobutylethyl-isoluminol (E_3-6-CMO-ABEI)

Assay procedure. Standard solutions were 0, 8.7, 17.4, 34.7, 69.4 and 138.8 nmol/l. The immunoassay was performed by mixing serum (0.02 ml) and phosphate buffer (0.02 ml, 0.01 M, pH 6.2) or estriol standard material in the same buffer (0.02 ml) and estriol-free serum (0.02 ml). Tracer solution (0.1 ml) and 0.5 ml of a suspension of anti-estriol antibodies attached to the solid particles ("Amerlex") were added. After incubation time, centrifugation and washing of the precipitate, the chemiluminescence of the precipitate was measured. The detailed immunoassay procedure is shown in the flow scheme (Fig. 2).

Light measurements. Measurements of light emission were performed by adding 0.1 ml of borate buffer (0.075 M, pH 8.6), 0.05 ml sodium hydroxide solution (2M) and microperoxidase solution to the precipitate. Just prior to measurement, 0.1 ml of hydrogen peroxide solution was added. Light measurements were started during this addition and calculated as integrals over a period of 30 seconds.

RESULTS

Yield of light of ABEI and estriol-6-CMO-ABEI. When the light emission of increasing quantities of ABEI and estriol-

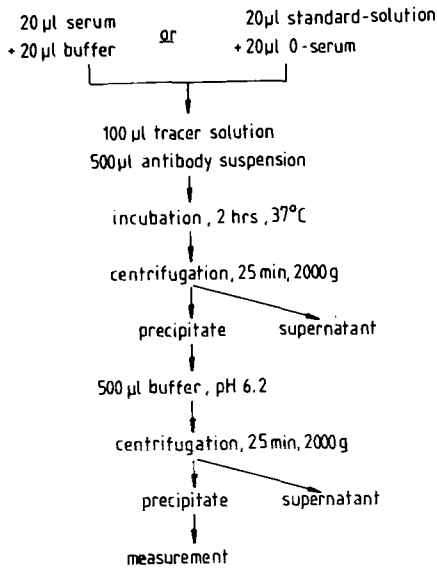

Fig. 2: Flow scheme of the chemiluminescence immunoassay for unconjugated estriol.

6-CMO-ABEI were measured, linearities over a wide range were shown (Fig. 3). The detection limit of ABEI was 0.4 ± 0.15 fmol (SD, $p < 0.05$), of estriol-6-CMO-ABEI was 1 ± 0.38 fmol (SD, $p < 0.05$).

Table 1: Influence of serum on light emission. E_3-6-CMO-ABEI (150 fmol) were dissolved in 0.1 ml phosphate buffered saline (0.06 M, pH 7.4) or five different sera, respectively.

Source	Photoncounts
tracer in protein-free buffer (pH 7.4)	51273
tracer in serum 1	52339
tracer in serum 2	51911
tracer in serum 3	50997
tracer in serum 4	51772
tracer in serum 5	52196

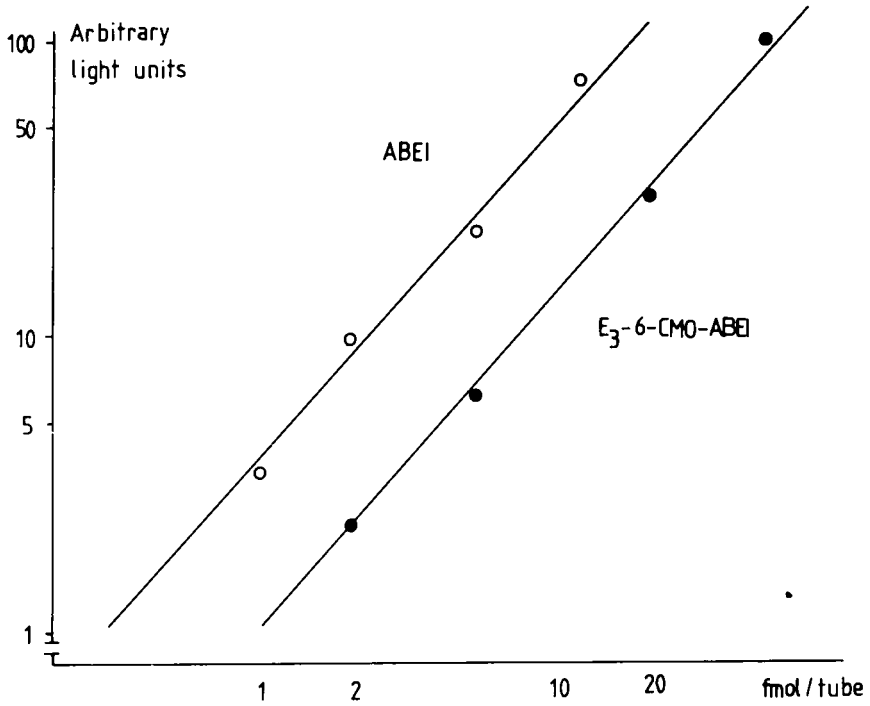

Fig. 3: Linearity of light measurements of increasing quantities of ABEI and E_3-6-CMO-ABEI, respectively.

Influence of serum components on luminescence measurement.

Light measurements of 0.1 ml of five different sera containing steroid chemiluminescent marker conjugate are shown in table 1. The serum influences are minimal and significant differences between the light emissions of the five sera were not found. On the other hand, solid particles of the antibody suspension decrease the light signal to a third of the original light emission. Because of the identical quantity of solid particles in each tube, this does not influences the results of the assays.

Performance of the assay. The reactions were carried out with varying quantities of unconjugated estriol dissolved in phosphate buffer as described in the experimental section. With increasing amounts of unconjugated estriol light emission decreases. Calibration curves were established from 8.7 to 138.8 nmol/l. Table 2 shows a representative dose response curve.

Table 2: Typical estriol chemiluminescence immunoassay data. Light emission units represents photon counting. Blanks are prepared in the same manner as the maximal binding samples, but instead of antiserum buffer was used. For further details see Experimental section.

Assay tube	Photon counts	Mean	Mean-Blank	Binding (%)
Total	110 271			
	109 390			
	111 176			
Blank	870	881		
	902			
	870			
Maximal	27 307	27146	26265	100
	26 408			
	27 723			
8.7 nmol/l	20 160	20290	19409	73.9
	20 053			
	20 658			
17.4 nmol/l	15 930	15983	15102	57.5
	16 457			
	15 561			
34.7 nmol/l	12 574	12399	11518	43.9
	12 380			
	12 243			
69.4 nmol/l	8 832	8840	7959	30.3
	8 744			
	8 944			
138.8 nmol/l	6 651	6737	5856	22.3
	6 552			
	7 007			

Sensitivity. The minimum concentration of estriol that could be significantly distinguished from zero was calcula-

ted from three dose response curves. The value was 700±340 (SD) pmol/l ($p<0.05$).

Specifity of the antibody. The percentage cross-reactivity of related compounds and of steroids with high serum concentrations was determined by the method of Abraham (13). The results obtained with the described chemiluminescence immunoassay and with a commercial available radioimmunoassay ("Amerlex unconjugated estrial RIA", Amersham International, U.K.) are shown in Table 3.

Table 3: The cross reactions of different steroids expressed as % concentrations giving 50 % inhibition of labelled antigen bound to antibodies.

Steroid	Chemiluminescence immunoassay	Radioimmunoassay
estriol	100.0	100.0
estrone	< 0.1	< 0.1
estradiol	< 0.1	< 0.1
cortisol	< 0.1	< 0.1
17-hydroxyprogesterone	< 0.1	< 0.1
progesterone	< 0.1	< 0.1
estriol-3-sulphate	< 0.1	0.3
estriol-3-glucuronide	0.7	0.5
estriol-16α-glucuronide	< 0.1	< 0.1
6-oxo-estriol	229.0	188.2
17-epi-estriol	< 0.1	0.2
16-epi-estriol	1.8	4.0

Precision. Within assay and between assay precision results are shown in table 4.

Table 4: Within assay and between assay precision. For both studies two plasma pools (A and B) were used.

	Mean ± SD (nmol/l)	Coefficient of variation (%)	n
Within-assay A	20.2±1.7	8.4	10
Within-assay B	73.9±7.8	10.6	10
Between-assay A	21.5±1.8	8.4	5
Between-assay B	74.3±9.6	12.9	5

Accuracy. One serum from a patient of the 40th gestation week was diluted with different volumes of a male serum. The regression of the measured and the expected estriol concentrations was linear and the correlation coefficient was r = 0.94.

Different amounts of estriol were added to a male serum. The recovery is shown in table 5.

Clinical samples. Thirty-one serum samples from pregnant women taken at different gestation weeks were assayed for estriol by chemiluminescence immunoassay and radioimmunoassay using the same antibody suspension. The results of the two methods agreed well: r = 0.95, y = 5.17 + 0.74x (x = radioimmunoassay) (Fig. 5).

Estriol values in the serum of five patients with obvious normal pregnancies and one patient with improved deficiency of the placenta are shown in figure 4. In the serum of two patients with intrauterine death estriol was not detectable.

Table 5: Recovery of estriol in serum.

	Value (nmol/l)	Found (nmol/l)	Recovery (%)
Serum I	17.4	18.1	104.0
Serum II	34.7	31.2	89.9
Serum III	52.1	48.0	92.1

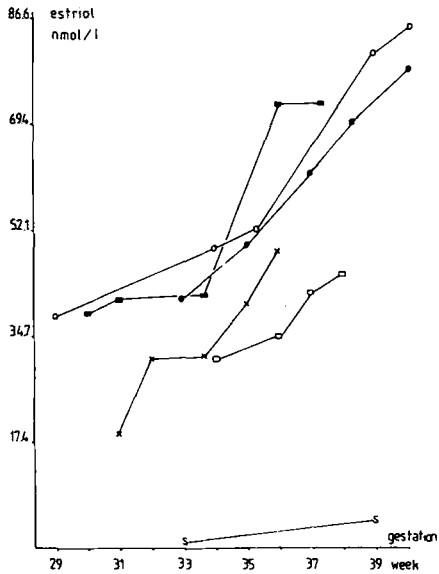

Fig. 4: Estriol values in the serum of five patients with obvious normal pregnancies and one patient (S-S) with improved sulphatase deficiency of the placenta.

DISCUSSION

The synthesis of estriol-6-CMO-ABEI is described for the first time. It was chemically prepared by coupling E_3-6-CMO with aminobutylethylisoluminol. The steroid-ABEI conju-

gate proved to be an excellent tool for chemiluminescence with a detection limit of 1 fmol under the reaction conditions used. In ethanolic stock solutions the estriol-6-CMO-ABEI can be stored at 4°C in the dark for at least 2 years without significant loss of activity.

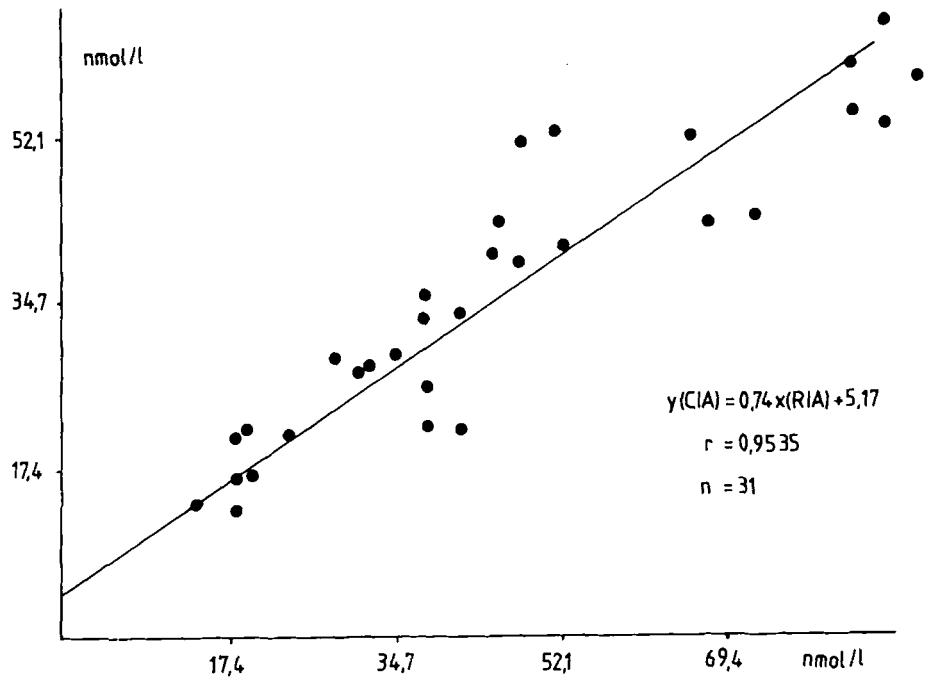

Fig.5: Comparison of serum estriol levels determined by radioimmunoassay and chemiluminescence immunoassay.

The estriol-6-CMO-ABEI enabled us to develop a solid-phase immunoassay for serum unconjugated estriol based on chemiluminescence. The assay method requires three steps: (i) incubation of antibodies with the marker conjugate and with serum or standards, (ii) separation by centrifugation, and

(iii) subsequent measurement of the light emission of the bound label by the oxidation with H_2O_2 using microperoxidase as catalyst. By introducing a washing step after the separation of bound and free antigens, the luminescence measurement is not affected by serum components.

Under the described conditions, standard curves for unconjugated estriol covering the range of 0.22-3.47 pmol/tube (8.7-138.8 nmol/l; 2.5-40 ng/ml) were obtained and the sensitivity of the assay was found to be 0.7 nmol/l. These results and the high specificity are comparable to common RIA methods. The precision of the chemiluminescence immunoassay is slighty inferior to that of the radioimmunoassay; this fact is probably due to the use of microperoxidase as catalyst.

There is a high correlation between the results of thirty one clinical samples determined by chemiluminescence immunoassay and by radioimmunoassay. The estriol concentrations found in serum of pregnant women with the non-isotopic method are lower, but this is not surprising. In external quality control programs, we have observed that our RIA method gives higher values than those of other laboratories in that study.

An estriol assay using the chemiluminescence method can

easily be performed within three working hours. The step of recording the light production was done manually. This is time consuming but meanwhile automatic equipments comparable to radioactivity counters are available (Berthold, Wildbad, FRG). Such kind of apparatus as well as the lack of radioactivity should make the chemiluminescence immunoassay attractive to numerous laboratories. It seems that, especially in the field of steroids, chemiluminescence will be a reasonable alternative to radioimmunoassay.

ACKNOWLEDGEMENTS

We thank Amersham International plc, U.K. and Amersham Buchler, FRG for providing us with solid-phase antiserum suspension. We thank Laboratorium Prof.Dr.Berthold, FRG for providing us with a Biolumat LB 9500 luminometer.

REFERENCES

1. Schöneshofer, M., ÄRZTL. LAB. 24, 94 (1978).
2. Klingler, W., NUCL.-MED. 21, 161 (1982).
3. Briggs, J., Elings, V.B. and Nicoli, D.F., SCIENCE 212, 1266 (1981).
4. Ullmann, E.F., Schwarzberg, M. and Rubenstein, K.E., J. BIOL. CHEM. 251, 4172 (1976).
5. Sayo, H. and Hosokawa, M., YAKUGAKU ZASSHI 100, 56 (1980).
6. Kohen, F., Kim, J.B., Lindner, H.R. and Collins, W.P., STEROIDS 38, 73 (1981).
7. Pazzagli, M., Kim, J.B., Messeri, G., Martinazzo, G., Kohen, F., Franceschetti, F., Tommasi, A., Salerno, R. and Serio, M., CLIN. CHIM. ACTA 115, 287 (1981).
8. Strasburger, C.J., Fricke, H. and Wood, W.G., FRESENIUS Z. ANAL. CHEM. 311, 351 (1982).
9. Schroeder, H.R., Yeager, F.M., Boguslaski, R.C. and Vogelhut, P.O., J. IMMUNOL. METHODS 25, 275 (1979).
10. Arakawa, H., Maeda, M. and Tsuli, A., CHEM. PHARM. BULL. 30, 3036 (1982).
11. Klingler, W., Haupt, O., von Postel, G. and Knuppen, R., ACTA ENDOCRINOL. (Kbh) Suppl. 246, 22 (1982).
12. Schroeder, H.R., Boguslaski, R.C., Carrico, R.J. and Buckler, R.T., METHODS ENZYMOL. 57, 424 (1978).

13. Abraham, G.E. ACTA ENDOCRINOL. (Kbh) 183, 1 (1974).
14. Kuss, E., Goebel, R. STEROIDS 19, 509 (1972).
15. Longwell, B., Wintersteiner, O., J. BIOL. CHEM. 133, 219 (1940).
16. The following trivial names are used: estriol = E_3 = 1,3,5(10)-estratriene-3,16α,17β-triol; estriol-sulphate = 1,3,5(10)estratriene-3,16α,17β-triol-3-sulphate; estriol-3-glucuronide = 1,3,5(10)estratriene-3,16α, 17β-triol-3-D-glucopyranosiduronic acid; estriol-16α-glucuronide = 1,3,5(10)estratriene-3,16α,17β-triol-16α-D-glucopyranosiduronic acid; estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol = 1,3,5(10)estratriene-3,17β-diol; cortisol = 11β,17,21-trihydroxy-4-pregnene-3,20-dione; progesterone = 4-pregnene-3,20-dione; 16-epi-estriol = 1,3,5(10)-estratriene-3,16β,17β-triol; 17-epi-estriol = 1,3,5(10)-estratriene-3,16α,17α-triol.